Molecular Genetic and Physiological Analysis of the Cold-Responsive Dehydrins of Blueberry

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SUMMARY. Blueberry plants, like many perennial plants, must undergo physiological changes in order to survive winter. Lack of winter hardiness and susceptibility to spring frosts have been identified as two

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of the most important genetic limitations of current blueberry cultivars. These traits are influenced by several factors such as the level of cold hardiness reached while plants are in the cold acclimated state and the chilling requirement or amount of low-temperature exposure required for breaking dormancy. How these factors are controlled genetically and how they interact with each other is not well understood. For these reasons, we have been using a combination of molecular, genetic, and physiological approaches to investigate genetic controls of chilling requirement, cold hardiness, and related factors in blueberry. Previously, we identified three dehydrins of 65, 60, and 14 kDa as the predominant proteins present in cold acclimated blueberry floral buds. Dehydrins are a group of heat-stable, glycine-rich plant proteins that are induced by environmental stimuli that have a dehydrative component, such as drought and low temperature. Levels of the blueberry dehydrins increase with cold acclimation and decrease with deacclimation and resumption of growth.

Expression studies with whole plants indicate that blueberry dehydrins are induced by cold stress in all organs examined including floral buds, leaves, stems, and roots, and by drought stress in primarily stems. Although dehydrin accumulation correlates positively with cold hardiness levels, it does not correspond precisely to the degree of drought tolerance or drought avoidance. Our studies of dehydrin expression in cell suspension cultures indicate that cell cultures are not a good system for studying blueberry dehydrin expression.

Peptide sequence information from the blueberry dehydrins has been used to prepare degenerate primers and amplify a portion of a gene encoding a dehydrin. This amplification product has been used to screen our cDNA library, prepared from RNA from cold acclimated blueberry floral buds, and has resulted in the isolation of a full-length cDNA clone thought to encode the 60 kDa dehydrin. The gene represented by this clone has been designated *bbdhn1*. This clone has now been used as a probe to further screen the cDNA library and has resulted, to date, in the isolation of four partial-length dehydrin cDNAs. All have been completely sequenced and the sequences compared to each other and to that of the *bbdhn1* cDNA. The sequences are identical at the 3′ end and diverge more and more as they approach the 5′ end. Whether the cDNAs represent different genes or a combination of different alleles and different genes remains to be determined. Efforts are currently underway to complete cloning and sequencing the remaining unique dehydrin cDNAs, as well as isolate and characterize cDNA clones representing other cold-responsive messages from blueberry. *[Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <docdelivery@haworthpress.com> Website: <http://www.HaworthPress. com> © 2004 by The Haworth Press, Inc. All rights reserved.]*

KEYWORDS. Blueberry, chilling requirement, cloning, cold acclimation, cold hardiness, cold-responsive genes, cold-responsive proteins, cold tolerance, dehydrins, dormancy, molecular biology, molecular genetics, physiology, *Vaccinium*

INTRODUCTION

Annually, woody perennial plants of the temperate zone must undergo physiological changes in order to survive winter. As part of this annual growth cycle, by late summer to mid autumn plants enter a state of dormancy known as endodormancy and begin to develop cold hardiness, referred to as the first stage of cold acclimation (Nissila and Fuchigami, 1978; Sakai and Larcher, 1987). Here, the prefix "endo" refers to the inhibition of bud growth which is maintained within the bud itself, as distinguished from paradormancy which is controlled by a factor outside the bud (for example, apical dominance), and ecodormancy, which is imposed by environmental factors (Lang, 1987; Lang et al., 1987). Endodormancy and this first stage of cold acclimation are thought to be triggered or enhanced by short photoperiods (Downs and Borthwick, 1957; Nitsch, 1957; Perry, 1971; Vince-Prue, 1975; Nooden and Weber, 1978). Endodormancy is characterized by a chilling requirement, i.e., exposure to an accumulated number of hours of low, nonfreezing temperatures in order for budbreak to occur the following spring (Lang et al., 1987). Thus, the chilling requirement serves to synchronize a plant's growth upon exposure to favorable environmental conditions. During winter months, while buds are fully endodormant and then ecodormant, there is a further increase in cold hardiness to reach maximum hardiness. This is the second stage of cold acclimation (Fuchigami et al., 1982). Some plants that would be killed by temperatures slightly below 0°C during the summer and early fall may survive temperatures as low as −196°C during the winter when fully cold acclimated (Sutinen et al., 1992). These physiological changes culminate, upon the return of warmer temperatures, in resumption of growth and fully deacclimated plants.

The United States is the world's leading producer of blueberries. In a survey of blueberry research and extension scientists in the United States, lack of winter hardiness and susceptibility to spring frosts were identified as the most important genetic limitations of current cultivars (Moore, 1993). The expression of these traits are due to the interaction of several components including how quickly plants cold acclimate in

the fall and deacclimate in the spring, the level of cold hardiness reached while plants are in the cold acclimated state, timing of plants entering and breaking dormancy, depth of dormancy, the chilling requirement, and the heat units required for resumption of growth in the spring. How these factors are controlled genetically and how they interact with each other is not well understood. One of the reasons for this is the difficulty in conducting genetic research in woody perennials. Factors such as long generation times, high levels of heterozygosity, and problems associated with inbreeding depression often render use of recombinant inbred lines and, sometimes, even true backcross and F_2 populations impossible for genetic and mapping studies. In terms of molecular genetic research, procedures that are routine in many herbaceous species, such as DNA, RNA, and protein extractions, often times are more difficult in woody plant species. Despite these difficulties and because of the importance of cold hardiness and dormancy-related traits to fruit crop industries, we are using a combination of molecular, genetic, and physiological approaches to investigate genetic controls of chilling requirement, cold hardiness, and related factors in blueberry.

This article will focus on describing molecular and physiological work related to the investigation of cold hardiness in acclimated blueberry. We will summarize our past work as well as present new findings. Our classical genetic and mapping work on control of chilling requirement and cold hardiness in blueberry has been recently reviewed elsewhere (Rowland et al., 1999).

IDENTIFICATION AND CHARACTERIZATION OF PROTEINS RESPONSIVE TO LOW TEMPERATURE IN BLUEBERRY

Identification of Chilling-Responsive Proteins in Floral Buds of Blueberry

Initially, to identify proteins responsive to low temperature exposure or "chilling," we examined changes in protein levels associated with chill unit accumulation in floral buds of two blueberry cultivars with different chilling requirements and levels of cold hardiness (Muthalif and Rowland, 1994a). The cultivars used were a high chilling, cold tolerant *Vaccinium corymbosum* L. cultivar 'Bluecrop' (chilling requirement of about 1200 chill units) and a lower chilling, more cold sensitive *V. ashei* Reade cultivar 'Tifblue' (chilling requirement of about 600

FIGURE 1. Profiles of total proteins from high chilling, cold tolerant *V. corymbosum* cultivar 'Bluecrop'. One hundred micrograms of total proteins, extracted from floral buds of field plants of 'Bluecrop' collected at various times during chill unit accumulation, were fractionated on a 12.5% gel by SDS-PAGE. Chill units are indicated by the number above each lane. In the far left lane are molecular mass markers. Arrows to the right mark the 65, 60, and 14 kDa polypeptides that accumulate with chilling.
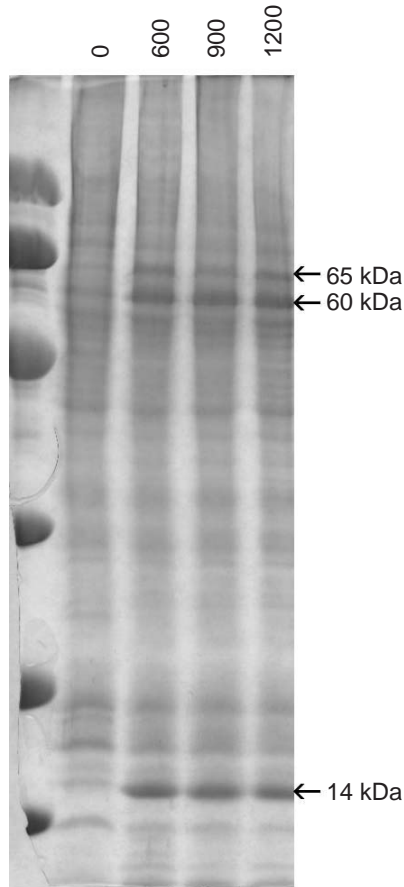
chill units). From profiles of total and soluble proteins (Figure 1), the levels of three proteins of 65, 60, and 14 kDa were observed to increase with chill unit accumulation such that they become the predominant proteins visible on one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gels in both cultivars (Muthalif and Rowland, 1994a,b).

The level of an additional 10 kDa protein was observed to increase in 'Tifblue' as well with chilling.

Densitometric scans of the protein gels indicated that levels of the proteins increased dramatically during the initial 300 chill units, reaching a maximum level by about 600-900 chill units. Protein levels declined sharply with exposure to higher temperatures and resumption of growth, reaching levels nearly equal to or below the 0-chill unit level by the time of budbreak. Additionally, the magnitude of the overall changes observed in 'Tifblue' were not as dramatic as those observed in 'Bluecrop', with the exception of the 10 kDa protein which was not detected in 'Bluecrop' (Muthalif and Rowland, 1994a).

Categorization of Chilling-Responsive Proteins as Dehydrins

Further characterization of the chilling-induced proteins revealed them to be members of a family of proteins known as dehydrins. Dehydrins are a group of heat-stable, glycine-rich plant proteins that are induced by environmental stimuli that have a dehydrative component including drought, low temperature, salinity, and seed maturation (Close, 1996). Also indicative of dehydrins is the presence of a highly conserved lysine-rich amino acid sequence (consensus sequence EKKGIMDKIKEKLPG) referred to as the K segment, which is often repeated several times (Close, 1996). The K segments are predicted to form amphipathic α-helices (Close, 1997) and amphipathic α-helices may have a role in stabilizing cell membranes against freezing damage (Thomashow, 1999). The categorization of chilling-responsive proteins in blueberry as dehydrins was based on several factors, including their reaction to antiserum raised against the dehydrin-specific consensus peptide or K segment, their boiling-stability, and the similarity in amino acid composition of selected sequenced peptides from the chilling-responsive proteins to dehydrins (Muthalif and Rowland, 1994a).

Association of Blueberry Dehydrins with Cold Acclimation

To determine if levels of the chilling-induced dehydrins were associated with levels of cold hardiness, freezing tolerance of floral buds of 'Bluecrop' and 'Tifblue' was determined about every 300 chill units and compared to protein profiles (Muthalif and Rowland, 1994a). Bud cold hardiness was evaluated using a laboratory controlled freeze-thaw regime, followed by a visual assessment of injury. Cold hardiness or

freezing tolerance (LT_{50}) was defined as the temperature causing 50% of the flower buds to be injured. For the two cultivars tested, levels of the dehydrins did appear to correlate with cold hardiness levels. The largest increase in the dehydrin levels (during the initial 300 chill units) coincided with the largest increase in the level of cold hardiness and the most dramatic decline in cold hardiness occurred with the resumption of growth, as did the decline in levels of the dehydrins. Also, maximum level of cold hardiness was higher in 'Bluecrop' (LT_{50} of $-29°C$ for field plants) than 'Tifblue' (LT_{50} of $-22°C$) as was maximum level of the dehydrins.

The most cold hardy cultivar in this initial study, 'Bluecrop', is also the cultivar with the highest chilling requirement. Since cold acclimation and development of dormancy, as well as deacclimation and release from dormancy, occur simultaneously in woody perennials, it is impossible from the work described above to conclude unequivocally that dehydrins are more closely associated with cold acclimation than with dormancy transitions. Consequently, in a follow-up study, we used a novel strategy to separate cold acclimation/deacclimation and dormancy transitions in blueberry floral buds in order to independently study the relationship of dehydrin expression to these two processes (Arora et al., 1997). In this study, greenhouse-grown plants of three cultivars, 'Bluecrop', 'Tifblue', and 'Gulfcoast' (*V. corymbosum*) were used, having chilling requirements of approximately 1400, 900, and 600 chill units, respectively, when assessed by chilling plants constantly at 4°C under controlled conditions (where 1 chill unit = 1 hour at 4°C). These chilling requirements are somewhat higher than those reported for field-grown plants of these cultivars (where 1 chill unit was defined as 1 hour of exposure to 0-7°C [Muthalif and Rowland, 1994a]). This is consistent with other studies comparing chilling requirements under natural versus artificial conditions.

In this study, the three cultivars were first exposed to 4°C for long enough to satisfy one-half of their respective chilling requirements. This treatment resulted in cold acclimation. A fraction of the dormant and cold hardy plants were then given a temperature treatment of 15°C day/12°C night for 2 weeks which resulted in deacclimation but was dormancy-neutral, i.e., did not negate the chill units already accumulated. The remainder of the plants continued storage at 4°C until and beyond satisfaction of their chilling requirements. Before and after each treatment, cold hardiness and dormancy status of floral buds were determined. In addition, proteins were extracted and analyzed by one-dimensional SDS-PAGE followed by immunoblotting with anti-dehydrin

antiserum. We found first that in plants given the deacclimation/dormancy-neutral treatment the dehydrin levels decreased to the pre-chilling level. Densitometric scans of protein gels indicated a good correlation between the abundance of dehydrins in cold acclimated plants and the degree of cold hardiness in the three cultivars. In addition, levels of the dehydrin proteins and cold hardiness levels remained about the same between 100% and >100% satisfaction of the chilling requirements. Taken together, these results indicated that changes in dehydrin levels are associated with cold hardiness transitions rather than with changes in dormancy status.

Effect of Cold and Drought Stress on Accumulation of Blueberry Dehydrins in Leaves, Stems, and Roots

To determine if the dehydrins are induced in other organs in response to low temperature treatment and in response to drought stress, accumulation of dehydrins was also examined in leaves, stems, and roots of various blueberry genotypes (*V. ashei* cultivar 'Climax' for low temperature treatments and *V. ashei* cultivar 'Premier' for drought treatments, and *V. corymbosum* cultivar 'Bluecrop' and *V. darrowi* Camp selection Fla4B for both low temperature and drought treatments) by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with antidehydrin antiserum (Panta et al., 2001). Cold treatment involved placing plants in a cold room maintained at 4°C for 5 weeks; drought stress was imposed by withholding water from potted, greenhouse-grown plants for over 30 days. Relative water content of shoots was determined periodically throughout the drought treatment.

Dehydrins were found to accumulate with both cold and drought stress but their molecular masses varied depending upon blueberry genotype. Major 65 and 60 kDa dehydrins accumulated in the *V. corymbosum* cultivar 'Bluecrop' and the two *V. ashei* cultivars, 'Climax' and 'Premier'. However, 60 and 54 kDa dehydrins accumulated in the *V. darrowi* selection Fla4B. The 14 kDa dehydrin accumulated to low levels in response to cold stress in 'Bluecrop' but did not appear to accumulate in response to drought stress in any of the genotypes.

Blueberry dehydrins accumulated to higher levels in floral buds, stems, and roots than in leaves with cold stress and to higher levels in stems than in either roots or leaves with drought stress. In the cold stress experiment described above, the level of dehydrin accumulation correlated with expected level of plant cold hardiness in the three genotypes examined. With drought stress, dehydrins accumulated prior to signifi-

cant changes in relative water content, and dehydrin levels did not appear to be closely correlated with relative water content either among or within genotypes, suggesting that dehydrins may not play a major role in determination of drought tolerance in blueberry and/or they may not respond to desiccation *per se* but, perhaps, to associated rises in ABA levels. This ABA-responsiveness, rather than desiccation responsiveness, has been shown to be the case with many drought-inducible proteins (Shinozaki and Yamaguchi-Shinozaki, 2000).

Effect of Photoperiod on Expression of Blueberry Dehydrins in Leaves and Stems

Although we do not have direct evidence for short photoperiods inducing accumulation of blueberry dehydrins, we do have some indirect evidence that supports this idea. In an experiment comparing the effect of cold treatment combined with total dark treatment to cold treatment combined with a 10 hour light/14 hour dark photoperiod on dehydrin induction in leaves and stems, dehydrins were induced to higher levels with the total dark treatment (Panta et al., 2001). This suggests that dehydrins may be responsive to changes in photoperiod. Decreasing photoperiod and temperature are the environmental signals for plant cold acclimation. Since dehydrins are associated with plant cold hardiness, a combination of cold and no light (or daylength shorter than 10 hours of light) might induce some dehydrins to a higher level than cold and 10 hours of light, as seen in stems and leaves. Also, in light, some leaf nitrogen might be diverted to photosynthetic proteins, while in dark, all available nitrogen can be used for other proteins. Alternatively, total darkness itself may constitute another type of "stress" that results in higher dehydrin levels. Whether the blueberry dehydrins respond to changes in photoperiod alone (in the absence of cold treatment) is unknown at this time.

Expression of Blueberry Dehydrins in Cell Suspension Cultures versus Whole Plants

Low temperature treatment (Hellergren, 1983; Wallner et al., 1986; Arora and Wisniewski, 1995) and ABA applied at warm temperatures (Tremblay et al., 1992) have been shown to result in cold acclimation of cell suspension cultures derived from some woody plants. Cell suspension culture systems offer some advantages over whole plants for gene expression studies. Treatment of whole plants with ABA can be prob-

lematic possibly due to inadequate uptake, rapid metabolism, and/or microbial degradation (Chen and Gusta, 1983). Besides allowing for more control over treatments with ABA and other chemicals, a cell suspension culture system for blueberry would allow experiments to be more easily repeated and to be carried out year-round without concern for differences in developmental or physiological stages of plants. Therefore, we examined the induction of dehydrins in blueberry cell suspension cultures derived from leaf and stem tissue in response to low temperature, ABA, and polyethylene glycol (PEG) treatments (Parmentier-Line et al., 2002).

By one dimensional SDS-PAGE followed by immunoblotting, we compared the induction of dehydrins with cold, ABA, and PEG treatments in cell suspension cultures of two cultivars, 'Gulfcoast' and 'Tifblue', to that seen with low temperature and drought treatments of whole plants (Parmentier-Line et al., 2002). Unfortunately, the response was quite different. Cold treatment of cell suspension cultures resulted in an increase in the level of a 30 kDa dehydrin rather than the major 65, 60, and 14 kDa dehydrins seen in whole plants. A 65 kDa dehydrin was detected in cell suspension cultures, as in whole plants, but its level did not change with cold treatment. Certain concentrations of ABA (10^{-5} and 10^{-6} M) resulted in increases in the levels of both the 65 and 30 kDa dehydrins. On the other hand, PEG treatment to induce water stress resulted in little change in the 65 or 30 kDa dehydrins of cell suspension cultures, providing further support for the idea that the blueberry dehydrins may not respond to desiccation itself but to increases in ABA levels associated with drought stress in whole plants. Regardless of the interpretation, however, because of the different responses from that seen in whole plants, and the difficulties in maintaining the cultures, we have concluded that cell suspension cultures are not a good system for studying dehydrin expression in blueberry.

Expression of Molecular Chaperones in Response to Low Temperature Stress in Blueberry

Members of another group of proteins called molecular chaperones have been identified in some annual plants as being responsive to low temperature stress. The primary function of molecular chaperones is to assist in the transport, folding, and assembly of other proteins (Ellis, 1990; Gething and Sambrook, 1992). Indeed, a chaperone-like function has been suggested as one possible function of the dehydrin family of proteins (Close, 1996). Examples of cold-induced molecular chaperones

that have been identified in other plants are the CAP 79 protein from spinach, which is a member of the HSP 70 family of proteins (Neven et al., 1992), and cyclophilin from maize (Marivet et al., 1992). We used antiserum raised against CAP 79 and a cyclophilin from *Arabidopsis* to determine whether immunologically-related proteins are induced in floral buds of blueberry during low temperature exposure (Muthalif and Rowland, 1995). CAP 79 antiserum reacted with a 70 kDa protein in blueberry floral buds; however, its abundance did not change with low temperature treatment. Cyclophilin antiserum reacted with a 17.5 kDa polypeptide, which did appear to increase slightly in abundance with low temperature exposure in floral buds of three different cultivars tested, 'Bluecrop' (1.9×), 'Tifblue' (1.3×), and 'Berkeley' (*V. corymbosum* cultivar) (2.8×).

ISOLATION AND DNA SEQUENCE ANALYSIS OF BLUEBERRY DEHYDRIN cDNAs

Purification and Sequencing of the 65, 60, and 14 kDa Dehydrins

The 65, 60, and 14 kDa dehydrins were gel-purified for sequencing following either isoelectric focusing in solution combined with one-dimensional SDS-PAGE (Figure 2) or one-dimensional SDS-PAGE alone of soluble proteins extracted from floral buds of blueberry plants that had received 600-900 chill units (Muthalif and Rowland, 1994a; Levi et al., 1999). N-terminal sequencing of the 60 and 14 kDa dehydrins revealed that both are blocked at the N-terminus. Therefore, in subsequent analyses, all the dehydrins were subjected to endoproteinase Lys-C digestion and selected peptides were sequenced. Table 1 summarizes the sequence data for all the peptides sequenced to date from the dehydrins.

Cloning a Full-Length cDNA That Encodes the 60 kDa Dehydrin

Peptide sequence information from the 65 and 60 kDa dehydrins of blueberry was used to synthesize degenerate primers for amplification of part of the gene(s) encoding these proteins (Levi et al., 1999). The amino acid sequences with the least codon degeneracy were chosen for synthesis of the degenerate primers. Because it was not known which peptide sequences were N-terminal and which were C-terminal, e.g., which of the primer sequences were 5′ and which were 3′, pairs of primers, assuming a 5′-3′ and 3′-5′ orientation, were used in polymerase chain reactions (PCRs). One pair of primers, based on amino acid se-

FIGURE 2. Comparison of profiles of soluble proteins, fractionated first by isoelectric focusing in solution and second by one-dimensional SDS-PAGE, from floral buds of *V. corymbosum* cultivar 'Berkeley' collected after 0 and 950 chill units. Isoelectric focusing was performed using 9 mg of proteins from each time point. Fractions 16-19 (from the basic end of the isoelectric focusing cell, pH 7.0-8.5) are shown. Because of the dramatic increase in the levels of the 65, 60, and 14 kDa dehydrins (indicated to the right of the gel), fractions from 950 chill unit buds were loaded at 1/5 (labeled as 950′-0.2x) and 1/10 (labeled as 950′-0.1x) the volume of the comparable 0 chill unit fractions. Proteins were separated through a 12.5% gel. It should be noted that isoelectric focusing varied slightly between the 0 and 950 chill unit samples. Proteins were shifted by one fraction toward the basic end in the 0 chill unit sample as compared with the 950 chill unit sample. Thus, fractions 17-19 of the 0 chill unit sample are comparable to fractions 16-18 of the 950 chill unit sample. Adapted from Muthalif and Rowland (1994b).
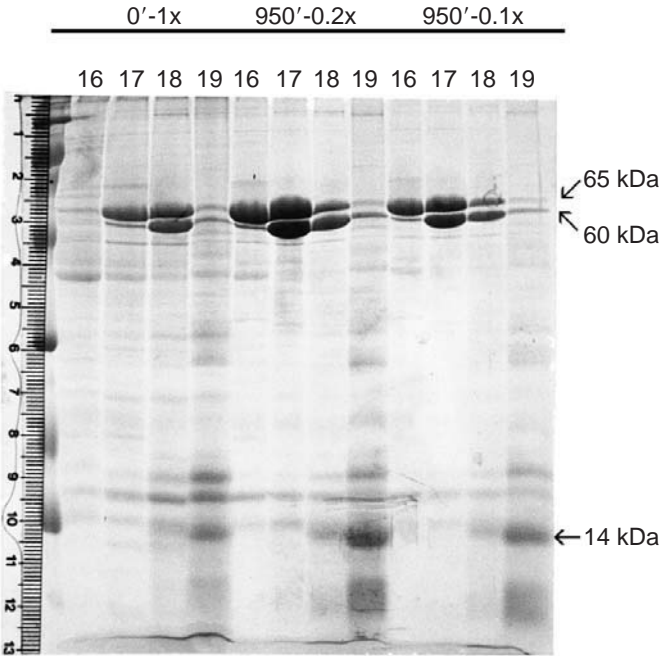
quences QDQLH and SADQNQ derived from the 65 kDa dehydrin, resulted in amplification of a 174 bp fragment. The fragment was cloned and sequenced. The presence of a K box (EGGGLADKVKDKIHG) within the sequence confirmed that part of a dehydrin gene had been cloned.

TABLE 1. Peptide sequences obtained after Lys-C digestion of the 65 and 60 kDa dehydrins of blueberry. The asterisk indicates a lower confidence secondary sequence. All others are high confidence primary sequences.

Dehydrin	Peptide Sequence
65 kDa	KQDQYRVG
	KEGGGLVDK
	KQDQLHGGYK
	KIHGGGG-SADQNQGGYK*
60 kDa	KEGGGLIYK
	KQDQLHGGYK
	KEGGGLMGELK
	KGGEQHQQQYNK
	KIHGGDGGSADQHQGIYGQDQQL–GY
14 kDa	K[S/A/I/K]GGGLMDK
	K[S/A/I/K]PGV[H/I][Y/G]GGAD[D/G]Q*

The 174 bp PCR fragment was used to screen a cDNA library prepared from RNA from dormant, cold-hardy floral buds of 'Bluecrop' (Levi et al., 1999). The buds for the cDNA library construction were collected from field plants having acquired approximately 600 chill units, since previously we showed that dehydrin levels are maximal by about 600-900 chill units (Muthalif and Rowland, 1994a). Hybridization with the 174 bp PCR fragment resulted in the isolation and purification of a clone with a 2.0 kb insert.

The 2.0 kb cDNA was sequenced and found to be full length. Inspection of the sequence confirmed that it encodes a member of the dehydrin family of proteins. Like dehydrins (Close, 1996), the deduced protein is hydrophilic, has a preponderance of glycine residues, is rich in polar and charged amino acids (such as glutamine, aspartic acid, lysine, tyrosine, histidine, glutamic acid, and arginine) and contains no phenylalanine or tryptophan. The deduced protein sequence contains

five lysine-rich repeats or K boxes indicative of dehydrins. These sequences are contained within larger contiguous imperfect repeats composed of 48-62 amino acids (consensus sequence for blueberry cDNA QDQQLGGYRQDQRKEGGGLMDKVKDKIHG GGGGSADQHQGGY (K/G)QDQQ(H/L)GGYR). The cDNA does not contain a tract of serine residues (S segment) or the consensus amino acid sequence (V/T) DEYGNP (Y segment) present in some dehydrins (Close 1996). In addition, a computer search of the GenBank database revealed similarity to other dehydrins, the following five being the highest scoring matches: alfalfa cold acclimation protein, CAS15 (Monroy et al., 1993); *Pistacia* inflorescence bud protein, 32 kDa (accession Y07600); spinach cold acclimation protein, CAP85 (Neven et al., 1993); peach dehydrin, PCA60 (Artlip et al., 1997); and citrus cold-stress protein, COR19 (Cai et al., 1995).

A sequence identical to the 174 bp sequence (amplified from degenerate primers derived from peptide sequences from the 65 kDa dehydrin and used as a probe to isolate this cDNA) is not present within the cDNA sequence. However, very similar sequences are present as part of the five large imperfect repeats. The five high-confidence peptide sequences, ranging from 9 to 25 amino acids long (Table 1), obtained from the 60 kDa dehydrin exactly match sequences encoded within the cDNA clone. Amino acid composition of the 60 kDa dehydrin also agrees well with the expected amino acid composition based on the cDNA sequence. Based on this information, we concluded that the 2.0 kb dehydrin cDNA encodes the 60 kDa dehydrin, and the gene represented by this clone was named as *bbdhn1*, for blueberry dehydrin 1.

Glycosylation of Blueberry Dehydrins

Our conclusion that the 2.0 kb dehydrin cDNA encodes the 60 kDa dehydrin was made despite the fact that the DNA sequence and coupled *in vitro* transcription/translation reactions of the clone followed by SDS-PAGE indicated that it encodes a dehydrin with a native molecular mass of ~40 kDa instead of 60 kDa (Levi et al., 1999). The most obvious explanation for this discrepancy is that the 60 kDa dehydrin undergoes extensive post-translational modification such as heavy glycosylation resulting in a higher molecular weight than that predicted from the DNA sequence or *in vitro* transcription/translation reactions alone. Indeed, previously, we had suspected that the 65 and 60 kDa dehydrins of blueberry were glycosylated because of their negative reaction to silver staining (Muthalif and Rowland, 1994b). To test this idea, we used a

commercially available glycoprotein detection system and showed that floral bud proteins of 65 and 60 kDa, which co-migrate with the 65 and 60 kDa dehydrins (detected with antidehydrin antibody) are glycosylated (Levi et al., 1999). To our knowledge, this is the first report of dehydrins being glycosylated. Whether other researchers have explored this possibility is unknown. The 2.0 kb dehydrin cDNA does not encode any of the Asn-XXX-Ser potential sites for N-linked glycosylation, but does encode six serine and three threonine residues, which could serve as potential sites of O-linked glycosylation.

Cloning Four Partial-Length Dehydrin cDNAs

The 2.0 kb blueberry dehydrin cDNA was then itself used as a probe to screen our cDNA library prepared from RNA from cold-hardened floral buds of 'Bluecrop'. In this screening, several positively hybridizing plaques were detected and, to date, four have been purified and completely sequenced. Analysis of the sequences confirm that they all are dehydrin cDNAs. All contain between two and five K boxes, all are hydrophilic, and all are rich in glycine and polar and charged amino acids such as glutamine. All are similar but unique-none of the sequences exactly match any of the other clones including *bbdhn1*. None, except for *bbdhn1,* are full length as their open reading frames do not begin with an ATG start codon. All four partial-length cDNAs have 3′-untranslated sequences which range in length from 196 to 224 bp, none of them being as long as the 714 bp 3′-untranslated sequence of *bbdhn1*. The four partial-length cDNA clones have inserts that are 0.6, 0.8, 0.9, and 1.2 kb long and the genes represented by these sequences were named *bbdhn2*, *bbdhn3*, *bbdhn4*, and *bbdhn5*, respectively. The cDNA sequences were entered into the GenBank and assigned accession numbers AF222738, AF222739, AF222740, and AF222741.

DNA and Predicted Protein Sequence Comparison of Blueberry Dehydrin cDNAs

To determine the degree of similarity among the cloned blueberry dehydrin cDNAs, DNA and predicted protein sequences were aligned using Vector NTI 5.0 software. The best alignment of the predicted protein sequences is shown in Figure 3. The alignments revealed that the five dehydrins are very similar at the DNA (data not shown) and protein levels. The sequences are more conserved at the 3′/carboxy end than at the 5′/amino end. In fact, all five cDNA clones have the same 61-amino

FIGURE 3. Comparison and best alignment of the predicted protein sequences of five dehydrin cDNAs isolated from blueberry. Sequences were compared using Vector NTI software. The numbers on top of the sequences represent total number of amino acid residues compared. The numbers that follow immediately after the cDNA name represent position of the amino acid relative to the beginning of the protein sequence. In addition, a consensus sequence, comprised of all amino acid residues found in the same positions of the alignment in a minimum of 3 of the 5 sequences, is shown below the sequences of the five clones. Amino acid residues of the individual clones that differ from the consensus sequence are indicated with bold lettering.

```
                    1                                                50
      BBDHN1    (1) --MAGIMNKIGQTLPCGGNKEEDKYKGGEQHQQQQYNKPGQHQGESRQDQ
      BBDHN2    (1) --------------------------------------------------
      BBDHN3    (1) --------------------------------------------------
      BBDHN4    (1) -------------RHEGGYKQD-----------------QQLGGHRQDQ
      BBDHN5    (1) TNSDCETANGSLNQHQGGYKQDQLHGGYRQDQQLGGHEQDQQRGGYRQDQ
   Consensus    (1)              GG K                    Q  G  RQDQ

                    51                                               100
      BBDHN1   (49) RKEGGGLMGELKDKIHGGGVGTDGGGVGSVDPHGHQGGKYRHDQQHSEYV
      BBDHN2    (1) --------------------------------------------------
      BBDHN3    (1) -----RHEDKVKDKIHGGGVGSVDQHQGGYKQDQLHGG-YRQDQQLGGHG
      BBDHN4   (20) HNEG--LADKVKDKIHGGGVGSADQHQGGYKQDQLHGG-YRQDQQLGGHG
      BBDHN5   (51) HNEG--LADKVKDKIHGGGVGSVDQHQGGYKQDQLHGG-YRQDQQLGGHG
   Consensus   (51)      EG   L DKVKDKIHGGGVGS DQHQGGYKQDQLHGG YRQDQQLGGHG

                    101                                              150
      BBDHN1   (99) QDQQPGGYRQDQRKEGGGLMDKVKDKIHGGGVGSADQHQDGYKQD-----
      BBDHN2    (1) --------------------RHEKIHGGGVGSADQHQDGYKQD-----
      BBDHN3   (45) QDQQPGGYRQDQHKEGGGLIYKVKDKIHGGDGGSADQHQGIYGQD-----
      BBDHN4   (67) QDQQRGGYRQDQHNEG--LADKVKDKIHGGGVGSADQHQGGYKQD-----
      BBDHN5   (98) QDQQRGGYRQDQHNEG--LADKVKDKIHGGGVGSADQHQGGYKQDQLLGG
   Consensus  (101) QDQQ GGYRQDQH EG   L DKVKDKIHGGGVGSADQHQGGYKQD

                    151                                              200
      BBDHN1  (144) ----QLHGGYKQDQQLGGYRQDQHKEGGGLIYKVKDKIHGGDGGSADQHQ
      BBDHN2   (24) ----QLHGGYKQDQQLGGHRQDQHNEG-----------------------
      BBDHN3   (90) ----QQLGGYRQDQQLGGYRQD--QQ------------------------
      BBDHN4  (110) ----QQPGGYGQDQQPGGYRQDQHKEG-----------------------
      BBDHN5  (146) YRQDQQPGGYGQDQQPGGYRQDQHKEG-----------------------
   Consensus  (151)     QQ GGY QDQQLGGYRQDQHKEG

                    201                                              250
      BBDHN1  (190) GIYGQDQQLGGYRQDQQLGGYRQDQQHGEYKQDQRKEGGGLMDKVKDKIP
      BBDHN2   (47) ---------------------------------------LADKVKDKIP
      BBDHN3  (110) ------------------------HGEYKQDQRKEGGWLMDKVKDKIP
      BBDHN4  (133) --------------------------------------GGLIYKVKDKIH
      BBDHN5  (173) --------------------------------------GGLIYKVKDKIH
   Consensus  (201)                                       GGLIDKVKDKIP

                    251                                              300
      BBDHN1  (240) GGNGGSAADQHQGVYGQDQQLGGYRQDQ----------------------
      BBDHN2   (57) GGNGGSAADQHQGVYGQDQQLGGYRQDQ----------------------
      BBDHN3  (134) GGNGGSAADQHQGVYGQDQQLGGYRQDQ----------------------
      BBDHN4  (145) GGDGG-SADQHQGIYGQDQQLGGYRQDQ----------------------
      BBDHN5  (185) GGDGG-SADQHQGIYGQDQQLGGYRQDQQLGGYRQDQQHGEYKQDQRKEG
   Consensus  (251) GGNGGSAADQHQGVYGQDQQLGGYRQDQ

                    301                                              350
      BBDHN1  (268) ---------------------------------------QRGEYKQDQR
      BBDHN2   (85) ---------------------------------------QRGEYKQDQR
      BBDHN3  (162) ---------------------------------------QRGEYKQDQR
      BBDHN4  (172) ---------------------------------------QRGEYKQDQR
      BBDHN5  (234) GWLMDKVKDKIPGGNGGSAADQHQGVYGQDQQLGGYRQDQQRGEYKQDQR
   Consensus  (301)                                        QRGEYKQDQR
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              351                                   387
BBDHN1    (278)  KEGGGLMDKVKDTIHGGAGGGADKHRGEYKQDQYRGD
BBDHN2     (95)  KEGGGLMDKVKDTIHGGAGGGADKHRGEYKQDQYRGD
BBDHN3    (172)  KEGGGLMDKVKDTIHGGAGGGADKHRGEYKQDQYRGD
BBDHN4    (182)  KEGGGLMDKVKDTIHGGAGGGADKHRGEYKQDQYRGD
BBDHN5    (284)  KEGGGLMDKVKDTIHGGAGGGADKHRGEYKQDQYRGD
Consensus (351)  KEGGGLMDKVKDTIHGGAGGGADKHRGEYKQDQYRGD
```

acid sequence at the carboxy end, suggesting that this sequence may serve an important function. As the sequences diverge toward the 5′/amino end, the dehydrins appear to differ from each other by a series of insertions/deletions and single base changes. It is possible that several of the cDNA clones represent alleles of each other rather than separate genes because 'Bluecrop', the cultivar from which these clones were isolated, is a tetraploid. A consensus K box for blueberry dehydrins, based on the sequences of all 20 blueberry K boxes that are encoded within the five dehydrin cDNAs isolated to date (shown in Figure 4), is comprised of 16 amino acid residues, EGGGLMDKVKDKIHGG. However, based on a closer examination of the K box sequences available so far, the blueberry dehydrin K boxes appear to fall into two basic types, one having the consensus sequence EGGGLMDKVKDKIHGG and the other having a more highly conserved sequence HNEGLADKVKDKIHGG. The dehydrin cDNAs analyzed so far appear to have either K boxes that are all of the EGGGLMDKVKDKIHGG type or a mixture of the EGGGLMDKVKDKIHGG and HNEGLADKVKDKIHGG types, with the EGGGLMDKVKDKIHGG types being found more toward the 3′/carboxy end. Of course, it is possible that other types will be found as more dehydrin cDNAs are isolated and sequenced.

Dehydrin Gene Copy Number in Blueberry

The number of dehydrin genes in blueberry has been estimated by hybridization of the *bbdhn1* probe to gel blots of genomic DNA of the original parent plants and a few F_1s of our diploid blueberry mapping populations (Levi et al., 1999). Genomic DNAs were digested with several different restriction enzymes, the sites of which were not present in the *bbdhn1* cDNA clone. Washes were carried out under either highly stringent or moderately stringent conditions. Each of the restriction enzyme digestions combined with highly stringent washes resulted in two or three strongly hybridizing fragments (not including allelic fragments, when possible to determine) and two to five weaker hybridizing fragments. Less stringent washes resulted in no increase in number of fragments hybridizing, but some of the weakly hybridizing fragments (from

FIGURE 4. The consensus blueberry dehydrin K segment along with a listing of the 20 individual K segments found in the five dehydrin cDNAs sequenced thus far. The number of times each amino acid in the consensus sequence occurs in the individual K segments is indicated in subscript.

Consensus																
	E_{14}	G_{14}	G_{14}	G_{17}	L_{19}	M_{10}	D_{15}	K_{19}	V_{19}	K_{20}	D_{20}	K_{15}	I_{20}	H_{16}	G_{20}	G_{20}
	H_5	N_5	E_5	W_2		A_5	Y_4	E_1	L_1			T_5		P_4		
						I_4	G_1									

BBDHN1	E	G	G	G	L	M	G	E	L	K	D	K	I	H	G	G
	E	G	G	G	L	M	D	K	V	K	D	K	I	H	G	G
	E	G	G	G	L	I	Y	K	V	K	D	K	I	H	G	G
	E	G	G	G	L	M	D	K	V	K	D	K	I	P	G	G
	E	G	G	G	L	M	D	K	V	K	D	T	I	H	G	G

BBDHN2	H	N	E	G	L	A	D	K	V	K	D	K	I	P	G	G
	E	G	G	G	L	M	D	K	V	K	D	T	I	H	G	G

BBDHN3							D	K	V	K	D	K	I	H	G	G
	E	G	G	G	L	I	Y	K	V	K	D	K	I	H	G	G
	E	G	G	W	L	M	D	K	V	K	D	K	I	P	G	G
	E	G	G	G	L	M	D	K	V	K	D	T	I	H	G	G

BBDHN4	H	N	E	G	L	A	D	K	V	K	D	K	I	H	G	G
	H	N	E	G	L	A	D	K	V	K	D	K	I	H	G	G
	E	G	G	G	L	I	Y	K	V	K	D	K	I	H	G	G
	E	G	G	G	L	M	D	K	V	K	D	T	I	H	G	G

| BBDHN5	H	N	E	G	L	A	D	K	V	K	D	K	I	H	G	G
	H	N	E	G	L	A	D	K	V	K	D	K	I	H	G	G
	E	G	G	G	L	I	Y	K	V	K	D	K	I	H	G	G
	E	G	G	W	L	M	D	K	V	K	D	K	I	P	G	G
	E	G	G	G	L	M	D	K	V	K	D	T	I	H	G	G

stringent washes) hybridized more strongly under these conditions. These results, along with the protein and peptide sequence data, suggest that blueberry dehydrins are encoded by a multigene family, with about two to three genes with high homology to the *bbdhn1* cDNA clone and a few other less related genes.

Accumulation of Dehydrin Transcripts in Response to Low Temperature

The *bbdhn1* cDNA clone, which encodes the 60 kDa dehydrin, and the 174 bp PCR fragment that was amplified from degenerate primers based on peptide sequences from the 65 kDa dehydrin have both been

used as probes on RNA blots to monitor the correlation in dehydrin mRNA levels in blueberry field plants with chill unit accumulation (Levi et al., 1999; Rowland et al., 1999). Both probes hybridized to two chilling-responsive messages of 2.0 and 0.5 kb (Figure 5), the 2.0 kb message being the same size as the *bbdhn1* cDNA. Consequently, it seems reasonable to conclude that the 2.0 kb size class of messages actually represents two messages, one encoding the 65 kDa dehydrin and one encoding the 60 kDa dehydrin, and that the 0.5 kb message encodes the 14 kDa dehydrin, as it is of an appropriate size to encode that dehydrin. RNA blots comparing dehydrin message levels in the more cold hardy cultivar 'Bluecrop' to that in the less hardy cultivar 'Tifblue' (Figure 5) revealed that, in both cultivars, the levels of both the 2.0 and 0.5 kb transcripts increased noticeably as early as 50 chill units, as compared with the 0 chill unit levels. However, the message levels reached maximum more quickly in the hardier cultivar 'Bluecrop' (by 300 chill

FIGURE 5. Northern blot of total RNA extracted from blueberry cultivars 'Tifblue' and 'Bluecrop' and hybridized with the 2.0 kb *bbdhn1* cDNA probe. RNA for northern was extracted from floral buds collected from field plants after different lengths of chilling (from 0 to 1500 chill units). Chill units are given above each lane. The probe hybridized to two messages of 2.0 and 0.5 kb, indicated by arrows to the right of the autoradiogram. Adapted from Rowland et al. (1999).
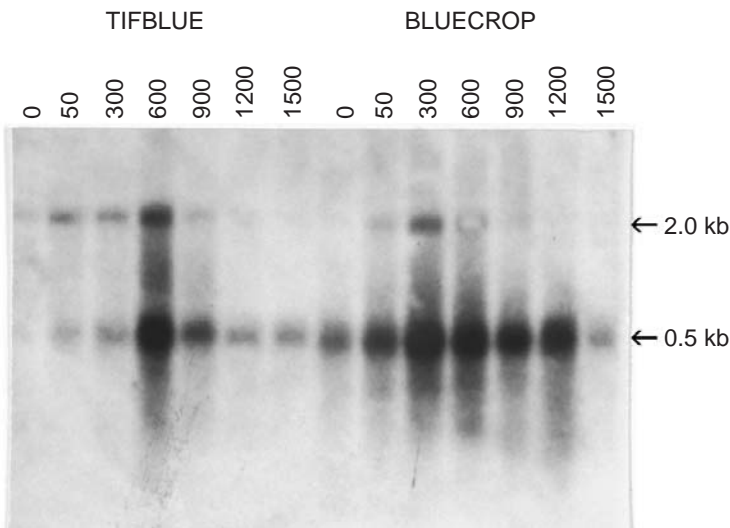
units) than in 'Tifblue' (by 600 chill units). In addition, the level of the 0.5 kb message remained higher for longer in 'Bluecrop' than in 'Tifblue', not declining dramatically until resumption of growth in the spring. The overall maximum level of the 2.0 kb message was about the same in both cultivars, whereas maximum level of the 0.5 kb message was about two fold higher in 'Bluecrop' than in 'Tifblue'.

In comparison to RNA accumulation, the 65, 60, and 14 kDa dehydrin proteins accumulated to higher levels quickly and remained at higher levels longer in 'Bluecrop' than in 'Tifblue' (Muthalif and Rowland, 1994a). The maximum level of all three proteins was higher in 'Bluecrop' than in 'Tifblue', although the largest difference seen was in the level of the 14 kDa dehydrin. The lag in reaching maximum protein levels (600-900 chill units) in 'Bluecrop' as compared to RNA levels (300 chill units) suggests that the dehydrin proteins are quite stable. This lag was not seen in 'Tifblue', where protein and RNA levels both peaked at about 600 chill units. Thus, the dehydrins of 'Bluecrop' may be more stable than those of 'Tifblue'. If dehydrins prove to play a causal role in determination of cold hardiness in blueberry, then the difference in hardiness between 'Bluecrop' and 'Tifblue' could be explained by a combination of earlier expression, overall higher expression (especially for the 14 kDa dehydrin), and greater stability of the dehydrins in 'Bluecrop' than in 'Tifblue'.

CONCLUSIONS

A recognized problem in the blueberry industry is the susceptibility of cultivars to freezing damage (Moore, 1993). Depending upon the geographical area, this type of damage may be caused by extreme low temperatures occurring during the winter while plants are still dormant, or by late winter or early spring frosts occurring while plants are beginning to resume growth. Therefore, a blueberry cultivar needs to have an appropriate chilling requirement and level of cold hardiness for the area in which it is intended to be grown. A cultivar with a chilling requirement that is too low for a particular area will resume growth too soon if exposed to fluctuating winter temperatures, making it susceptible to late frost damage. Conversely, a cultivar with a chilling requirement that is too high for a particular area may receive insufficient chilling resulting in delayed and erratic budbreak. Similarly, midwinter-freezing injury may occur if a cultivar is not cold hardy enough for certain growing areas.

Application of molecular genetic techniques to the study of cold hardiness and other dormancy-related traits should result in the identification of genes controlling these traits and to a better understanding of the role of these genes in control of cold hardiness, chilling requirement, etc. Once the genes are identified, theoretically, the level of cold hardiness or chilling requirement of a particular cultivar could be lowered or raised by over-expression or under-expression of the appropriate genes.

Our studies using blueberry floral buds have indicated that three major dehydrins of 65, 60, and 14 kDa increase with cold acclimation and decrease during deacclimation and resumption of growth. Furthermore, it has been shown that dehydrin expression in blueberry is closely associated with level of cold hardiness. More recently, the aim of our work has been to clone and sequence the cDNAs encoding the blueberry dehydrins, compare the sequences to each other and to dehydrins found in other plants, and further study the expression of the dehydrins at the RNA and protein levels in response to cold and drought stress in various organs of whole plants and in cell suspension cultures.

From proteins gels, peptide sequence data, and Southern blot data using one full-length 2.0 kb dehydrin cDNA as a probe, it appears that blueberry dehydrins are encoded by about 2-3 genes with high homology to the 2.0 kb cDNA, and a few other less related genes. In total, to date, one full length and four partial-length unique dehydrin cDNAs have been cloned and sequenced. To distinguish among them, they have been named, beginning with the full length clone, as *bbdhn1* (for *blueberry dehydrin 1*), *bbdhn2*, *bbdhn3*, *bbdhn4*, and *bbdhn5*. Based on peptide sequences encoded within the clone, we have concluded that the full-length *bbdhn1* cDNA encodes the 60 kDa dehydrin. Although dehydrin sequences are not colinear, e.g., short consensus sequences are present, but sequences outside the consensus are divergent in terms of length and sequence, a search of the GenBank database for sequences homologous to the *bbdhn1* cDNA has been performed. Highest scoring matches are to other dehydrins, alfalfa cold acclimation protein CAS15 and a 32 kDa *Pistacia* inflorescence bud protein. A comparison of the blueberry dehydrin clones reveal that there is very high homology both at the DNA and protein levels among the clones, particularly at the 3′/carboxy ends, and all contain multiple copies of the highly conserved K boxes. It is possible that several of the clones represent alleles of each other rather than separate genes.

RNA blots using RNA extracted from various organs, including floral buds, leaves, and stems, and the *bbdhn1* cDNA as a probe reveal homology to two chilling/cold-responsive messages of 2.0 and 0.5 kb. Our interpretation is that the 2.0 kb size class of messages actually represents two messages, one encoding the 65 kDa dehydrin and one encoding the 60 kDa dehydrin, and that the 0.5 kb message likely encodes the 14 kDa dehydrin. In addition, expression of the blueberry dehydrins in whole plants has been compared to that in cell suspension cultures. Cold and drought stress protein expression studies using whole plants indicate that blueberry dehydrins are induced by cold stress in all organs examined including floral buds, leaves, stems, and roots, and by drought stress in primarily stems. Furthermore, although dehydrin accumulation correlates positively with cold hardiness levels, it does not correspond precisely to the degree of drought tolerance or drought avoidance. For the most part, expression of the blueberry dehydrins in cell suspension cultures does not mimic that seen in whole plants. For example, cold treatment of cell suspension cultures results in an increase in the level of a 30 kDa cross-reacting protein rather than the major 65, 60, and 14 kDa dehydrins seen in whole plants. A 65 kDa dehydrin is detected in cell suspension cultures, as in whole plants, but its level does not change with cold treatment. Because of these differences and others, we have concluded that cell suspension cultures are not a good system for studying blueberry dehydrin expression.

Finally, our current efforts are focused in two areas. One area is to establish if the dehydrins, or other cold-responsive genes that we isolate, play a causal role in determination of cold hardiness in blueberry. To do this, collaborative efforts with other scientists are underway to use gene constructs of the *bbdhn1* cDNA in transformation experiments to determine their effect(s) on cold hardiness. Also, we are mapping the dehydrin genes to determine if any are associated with QTLs that control cold hardiness that we identify in our concurrent mapping work. The second area is to isolate and characterize other unique dehydrin cDNAs, as well as cDNA clones representing other cold-responsive messages from blueberry. To date, the only cold-responsive proteins that have been characterized in blueberry are the dehydrins and a cyclophilin. Because our own genetic studies with blueberry indicate that cold hardiness is a multigenic trait controlled largely by additive gene effects and, to a lesser extent, by dominance gene effects (Arora et al., 2000), we expect other genes, in addition to the dehydrins, to be involved in determination of cold hardiness.

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